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Salt-tolerant rootstock increases yield and photosynthesis of pepper under salinity through maintenance of root strength and proline accumulation

Consuelo Penella[a], Marco Landi[b], Lucia Guidi[b], Sergio G. Nebauer[c], Elisa Pellegrini[b], Alberto San Bautista[c], Damiano Remorini[b], Cristina Nali[b], Salvador López-Galarza[c], Angeles Calatayud[a,*]

[a]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultural. Ctra. Moncada-Naquera km. 4,5. 46113- Moncada, Valencia, Spain.*

[b]*Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80 - 56124 Pisa (Italy).*

[c]*Universitat Politècnica de València. Departamento de Producción Vegetal. Camino de Vera 14, 46020 Valencia, Spain.*

*Corresponding author: Angeles Calatayud. Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultural. Ctra. Moncada-Naquera km. 4.5. 46113, Moncada, Valencia, Spain.. Tel.: +34 3424039

E-mail addresses: penella_con@gva.es (C. Penella), marco.landi@for.unipi.it (M. Landi), lucia.guidi@unipi.it (L. Guidi), sergonne@bvg.upv.es (S.G. Nebauer), elisa.pellegrini@for.unipi.it (E. Pellegrini), asanbau@prv.upv.es (A. San Bautista), damiano.remorini@unipi.it (D. Remorini), cristina.nali@unipi.it (C. Nali), slopez@prv.upv.es (S. López-Galarza), calatayud_ang@gva.es (A. Calatayud).

ABSTRACT

The performance of a salt-tolerant pepper (*Capsicum annuum* L.) accession (A25) utilized as a rootstock was assessed in two experiments. In a first field experiment under natural salinity conditions, we observed a larger amount of marketable fruit (+75%) and lower Blossom end Root incidence (-31%) in commercial pepper cultivar Adige (A) grafted with A25 (A/A25) compared with ungrafted plants. In order to understand this behavior a second greenhouse experiment was conducted to determine growth, mineral partitioning, gas exchange and chlorophyll *a* fluorescence parameters, antioxidant systems and proline content in A and A/A25 plants under salinity conditions (80 mM NaCl for 14 days). Salt stress induced significantly stunted growth of A plants (-40.6% of leaf dry weight) compared to the control conditions, while no alterations were observed in A/A25 at the end of the experiment. Accumulation of Na^+ and Cl^- in leaves and roots was similar in either grafted or ungrafted plants. Despite the activation of protective mechanisms (increment of superoxide dismutase, catalase, ascorbate peroxidase activity and non-photochemical quenching), A plants showed severely reduced photosynthetic CO_2 assimilation (-45.6% of A_{N390}) and substantial buildup of malondialdehyde (MDA) by-product, suggesting the inability to counteract salt-triggered damage. In contrast, A/A25 plants, which had a constitutive enhanced root apparatus and 2.6-fold higher proline content under salinity, did not show alterations in photosynthesis and growth and MDA levels increased only slightly. Our results underline that salt tolerance in A/A25 grafted plants could be mediate by (I) the maintenance of root sink strength and (II) the markedly increased proline levels that could

balance cell osmotic pressure thus protecting enzymatic stability from salt-triggered damage.

Keywords: Antioxidant systems; *Capsicum annuum*; Chlorophyll fluorescence; Grafting; NaCl stress; Proline

1. Introduction

Nowadays, about 7% of the world`s land area and 20% of irrigated land are affected by salinity (Ferreira-Silva et al., 2010). In general terms, effects of salinity on plants are the result of both water stress (due to a higher osmotic potential in soil as compared to plant tissues) and a toxic effect caused by the influx of ions mainly Na^+ and Cl^- into plant tissues (Tuteja, 2007; Munns and Tester, 2008). The result of these effects is a wide range of physiological, metabolic and genomic changes that provoke alterations in photosynthesis, carbohydrate partition, respiration, increased reactive oxygen species (ROS) production, and an unbalanced uptake of other nutrients (Parida and Das, 2005; Hu and Schmidhalter, 2005; Chaves et al., 2009). Overall, the physiological changes induced by salinity correspond to diminished plant growth and yields.

In spite of these deleterious effects, plants present different degrees of tolerance to salinity, conferred by biochemical pathways, which can alleviate the negative effect of salt toxicity; amongst them: (I) retention and acquisition of water mediated by osmotically-active metabolites (mainly proline, glycine-betaine or sugars) (Singh et al., 2014); (II) maintenance of ion homeostasis (Rivero et al., 2014; Razzaghi et al., 2015); (III) induction of antioxidant systems (Ashraf et al., 2012; Hu et al., 2012; Wang et al., 2012; Fini et al., 2014); (IV)

over production of hormones (Krasensky and Jonak, 2012; Yoshida et al., 2014) or (V) synthesis of specific stress-associated molecules such as heat-shock proteins (Wang et al., 2004; Krasenski and Jonak, 2012; Pérez-Salamò et al., 2014) and late embryogenesis abundant proteins (Parida and Das, 2005, Radíc et al., 2013). In view of the complexity of salinity tolerance, differences on salt sensitivity occur not only among species, but sometimes even genotypes belonging to the same species perform differently under salinity (Shabala and Munns, 2012).

Pepper is one of the most important crops in Mediterranean area, which is usually classified as a salt-sensitive species (Kurunc et al., 2011; del Amor and Cuadra-Crespo, 2011), even though Aktas et al. (2006) observed that salt tolerance can vary amongst pepper genotypes. A promising perspective to improve pepper resistance to salinity is the use of grafting of commercial cultivars onto salt-tolerant rootstocks (Penella et al., 2013; Penella et al., 2015). The main general objective of using rootstocks is to increase scion growth and development rate, yield and fruit quality (Venema et al., 2008). Tomato and melon are the two commonest herbaceous species in which the grafting practice has been efficiently applied to obtain salt-tolerant plants (Estañ et al., 2005, Edelstein et al., 2011, Orsini et al., 2013). In melon, the favorable effects of grafting on plant growth cannot be ascribed to a more efficient exclusion of Na^+ or enhanced nutrient uptake but they were associated with a more efficient control of stomatal functions (changes in stomatal index and water relations), which may indicate that the rootstock may alter hormonal signalling between root and shoot (Orsini et al., 2013). As far as we know, very few studies on

grafted pepper plants have been conducted to elucidate whether or not salt tolerance might be conferred by rootstocks.

Given the poor genetic basis of cultivated pepper accessions, the screening of wild pepper accessions has been performed in previous works to assess naturally-occurring genetic variation to salinity in order to select salt-tolerant accessions to be used as rootstocks (Penella et al., 2014). In a previous work, a wild-type pepper accession (code A25) was select as high salt tolerant. Now, in this study, we used a valid commercial cultivar Adige either ungrafted (A) or grafted onto the rootstock A25 (A/A25) and we found an increased fruit yield under salinity conditions as compared with ungrafted plants. To gain insight into the mechanisms by which the grafting improved plant's yield, we addresses the question whether or not the increase of the production in these plants was associated with the maintenance of their photosynthetic capacity, ion homeostasis, osmotic regulation and/or water relations under 80 mM NaCl for 14 days. Gas exchange and chlorophyll fluorescence parameters, antioxidant systems, hydric and osmotic relations, and Na^+ and Cl^- partitioning were assessed to this aim.

2. Materials and methods

2.1 Plant material

Based on previous studies, a pepper accession of *Capsicum annuum* L. from the COMAV Genebank at the UPV university (Valencia, east Spain) was selected, which was tolerant to salinity (code A25). This accession was chosen to be used as a rootstock and pepper cultivar 'Adige' (A) (Lamuyo type, Sakata Seeds, Japan) was the scion. Seeds of A25 were sown in 96-hole seed trays

filled with an enriched substrate for germination. After 2 months, A plants were grafted onto A25 (A/A25). The graft was performed by the tube-grafting method (Penella et al., 2015). The ungrafted 'Adige' (A) plants were sown 2 weeks later to obtain plants with a similar biomass to that of the grafted plants at the time of transplantation (10-12 true leaves). The plants obtained by the aforementioned procedure were utilized for both field and greenhouse experiments.

2.2. Soil-field experiment

A preliminary experiment was conducted in spring/early summer 2013 in a field with soil with a moderate salt concentration (pH=8.0; EC as saturated past was 6.64 dS m^{-1}; Sand= 76%). The electrical conductivity and pH of the irrigation water were 7.5 dS m^{-1} and 7.60, respectively, with 57.5 mM of Na^+ and 71.2 mM of Cl^-. Plant density was 2.5 plants m^{-2} in sandy soil (in polyethylene greenhouses). Fertilizers were applied at a rate of 200 Unit of Fertilizer (UF) N, 50 UF P_2O_5, 250 UF K_2O, 110 UF CaO and 35 UF MgO. A randomized complete block design was used with three replicates for A and A/A25, each of them consisting of 25 plants. There was no significant difference among replicates in production. Ripe fruits were harvested from the end of May to the end of July, and marketable and unmarketable fruits, mainly due to BER, were weighed.

2.3. Hydroponic greenhouse experiment

Seeds were sown on January 29th (2014) and the grafting for A/A25 performed on March 29th. After 3 weeks of acclimation, 30 plants of each combination (A and A/A25) were separated into two groups: controls (C) and

NaCl-treated plants (+NaCl). For salt treatment, 80 mM of NaCl were added to a half-strength Hoagland's solution (pH 6.5±0.1; EC 8.0 dS m^{-1}). Both groups were watered daily with excess half-strength Hoagland's solution (pH 6.5±0.1; EC 1.1 dS m^{-1}) to minimize salt accumulation in the substrate for the 14 d that the experiment lasted. Potted plants were grown under greenhouse conditions at the facilities provided by the University of Pisa (Pisa, Italy). Temperatures ranged between 8.7 °C and 22.9 °C during the day**,** and remained above 12 °C at night**.** Relative humidity (RH) was between 37.7% and 96.3%, with daily maximum photosynthetically active radiation (PAR) levels within the greenhouse range of 850-1530 µmol m^{-2} s^{-1} (directly provided by sunlight).

All the physiological measurements were taken on fully expanded mature leaves (third or fourth leaf from the shoot apex) at the end of the salt treatment period. Two independent physiological determinations were made on each replicate and plant combination, obtained from six plants per treatment and combination.

2.3.1. Biomass and ion determination

Plants were harvested after 14 d of treatment. Leaves and roots were separated and their fresh weight (FW) was recorded. For dry weight (DW) determinations, leaves and roots were dried at 70 °C for 72 h in a laboratory oven and then weighed. Leaves and roots were milled and digested with concentrated HNO_3. Na^+ and K^+ were measured with an atomic absorption spectrophotometer (Ultrospec 2100, Pharmacia). Chloride analysis was performed on the water extracts of dry materials. The sample (250 mg DW) was incubated in water at 60 °C for 30 min. Following centrifugation, the supernatant

was collected and Cl^- was determined in an ion cromatograph (DX-100 ion chromatograph Dionex^{TM}, Thermo Scientific).

2.3.2. Water potential and relative water content

The leaf water potential at pre-dawn (Ψ_w) and the relative water content (RWC) were measured on the leaves sampled before dawn by a standard methodology (Guidi et al., 2008).

2.3.3. Gas exchange and PSII photochemistry measurements

The net CO_2 assimilation rate, stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) in the saturating light (A_{N390}, i.e., at 800±28 μmol quanta $m^{-2}s^{-2}$ and 390 μmol CO_2 mol^{-1}) determinations were taken on fully expanded leaves (3rd- 4th leaf from the apex) at room temperature (RT) and 75% RH with a portable LI-COR 6400 (Li-Cor Inc.) infrared gas analyzer. In the same leaves, the response of light-saturated CO_2 assimilation to variable internal CO_2 concentrations (A/C_i curves) was measured as reported in Guidi et al. (2008). From the A/C_i curves, the following photosynthetic parameters were calculated according to Long and Bernacchi (2003): the apparent maximum carboxylation rate of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco), V_{cmax}, the maximum rate of the electron transport (J_{max}), which is equivalent to the ribulose-1,5-*bis*P (RuBP) regeneration rate, and use of triose-P (TPU).

The chlorophyll *a* fluorescence parameters were estimated from the measurements taken on the dark- (for 30 min) and light-adapted leaves (about 800 μmol $m^{-2}s^{-1}$) by IMAGING-PAM (Walz, Effeltrich, Germany). The maximum quantum efficiency of PSII was calculated as F_v/F_m = (F_m - F_0/F_m). The

operating quantum efficiency of PSII photochemistry, Φ_{PSII}, was calculated as $(F'_m - F')/F'_m$. The electron transport rate was calculated as ETR= 0.5 x Φ_{PSII} x PAR x 0.84 μequivalents m^{-2} s^{-1}. The photochemical quenching (q_P) factor was determined as $(F'_m - F')/(F'_m - F'_0)$. Non photochemical quenching (NPQ) was expressed as $F_m/F'_m - 1$, where F'_m was maximal fluorescence during a saturating flash of light of about 8000 μmol m^{-2} s^{-1}, and F'_0 was the minimal fluorescence estimated by the approach of Oxborough and Baker (1997) $F_0' = F_0/(F_v/F_m + F_0/F_m')$.

2.3.4. Leaf lipid peroxidation

Leaf lipid peroxidation was estimated with the malondialdehyde (MDA) concentration measurements taken by the thiobarbituric acid reaction, as reported in Penella et al. (2015).

2.3.5. Antioxidant enzymes

Antioxidant enzyme activities were measured in the fresh leaf material extracted with 1 mL of 100 mM potassium phosphate buffer (pH 7.0) that contained ethylenediamine tetra-acetic acid (EDTA). The extract was then centrifuged at 11000 x *g* at 4 °C for 15 min, and the supernatant was used for all the enzyme assays, while the protein determinations were performed with the Protein Assay Kit II (Bio Rad).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 560 nm, based on the inhibition of nitroblue tetrazolium (NBT) reduction by SOD (Beyer and Fridovich, 1987). One unit of SOD was defined as the enzymatic amount required to reduce the NBT reduction state by 50%. Catalase (CAT; EC

1.11.1.6) activity was measured at 270 nm by determining the rate of conversion of H_2O_2 into O_2 and water, as described by Cakmak and Marschner (1992). Catalase activity was expressed as µmol H_2O_2 per mg protein and per minute. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined following the H_2O_2-dependent oxidation of ascorbate (AsA) at 265 nm in a reaction mixture composed of 50 µM AsA, 90 µM H_2O_2, 50-100 µg proteins and 0.1 M phosphate buffer (pH 6.4) (Nakano and Asada, 1981). APX activity was corrected by subtracting the non-enzymatic H_2O_2-dependent AsA oxidation. APX activity was expressed as µmol AsA per mg protein and per minute.

2.3.6. Proline

Proline content was determined according to the method of Bates et al. (1973) with some minor modifications. Plant material (100 mg FW) was ground in an ice-cold mortar with 2 mL of 3% sulfosalicylic acid. Homogenates were centrifuged for 30 min at 10,000 x *g* at 4 °C. The supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and 1 mL of the filtrate was mixed with equal volumes of glacial acetic acid and of ninhydrin reagent (1.25 g ninhydrin, 30 mL of glacial acetic acid, 20 mL 6 M H_3PO_4), and was incubated for 1 h at 100 °C. The reaction was stopped by placing test tubes in ice-cold water. Samples were vigorously mixed with 2 mL toluene. After 20 min, the light absorption of the toluene phase was estimated at 520 nm, with toluene used for a blank. The proline concentration was determined with a standard curve and calculated on a FW basis.

2.3.7. Tocopherol and β-carotene determination

The amount of α-tocopherol and ß-carotene was determined by HPLC according to Döring et al. (2014). Thirty mg of leaves were homogenized in 3 mL of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. The supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and immediately analyzed. The analysis was performed at RT with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length) and methanol/ethylacetate (68/32, v/v) was used as the mobile phase (flow rate 1 mL min^{-1}). α-tocopherol and ß-carotene were detected at 280 nm and 445 nm, respectively. Pure authentic standards were used to quantify the α-tocopherol and ß-carotene content of each sample.

2.3.8. Ascorbic acid content

Total ascorbate (AsAt), dehydroascorbate (DHA) and reduced ascorbate (AsA) were determined as described by Degl'Innocenti et al. (2005), based on the method of Kampfenkel et al. (1995). The ratio between AsA and AsA total (AsA/AsAt) was reported.

2.4. Statistical analysis

The experiment was completely randomized and the results were subjected to a two-way ANOVA (Statgraphics Centurion for Windows, Statistical Graphics Corp.) with salt treatment and plant type as the variability factors. The data of marketable fruits and the percentage (angularly transformed) of the fruits affected by BER were subjected to a one-way ANOVA with plant type as the variability factor. Means (n=6; ± SE) were compared using Fisher's least significance difference (LSD) test at $P < 0.05$.

3. Results

3.1. Fruit yield

Adige grafted onto accession A25 (A/A25) gave the best response in marketable fruit yield associated with the lowest percentage of BER with significant differences with A plants (Table 1).

3.2. Ion partitioning

After 14 days of culture in the greenhouse, Na^+ (Fig. 1A, D) and Cl^- (Fig. 1B, E) increased in both roots and shoots under salinity (80 mM NaCl) in both plant types. The Cl^- concentration was higher in leaves (Fig. 1E) than in roots (Fig. 1B) (3.3 *vs.* 6.1 mg g^{-1} DW, respectively; $P<0.01$), while no differences were observed in Na^+ content (1.5 *vs*. 1.7 mg g^{-1} DW in roots and leaves, respectively; $P<0.01$). The K^+/Na^+ ratio was higher in leaves than in roots (4-fold; $P<0.001$), and was significantly lower in both plant organs when salinity was applied (Fig. 1 C, F).

3.3. Water potential

Leaf water potential (ψ_w) significantly decreased following NaCl treatment in both genotypes, and reached values of -0.22 and -0.32 MPa in A and A/A25, respectively (Fig. 2). However, no differences between the control and stressed plants in RWC were observed (Fig. 2, inside).

3.4. Gas exchange and chlorophyll fluorescence parameters

At ambient atmospheric CO_2 concentrations, salinity significantly lowered the net assimilation rate at light saturation (A_{N390}), but only in A plants, whereas no differences were observed in A/A25 between controls and salt-treated individuals (Table 2). The intercellular CO_2 concentration (C_i) lowered in the salt-treated leaves of A/A25, but no differences were observed in A. Stomatal conductance (g_s) decreased significantly in both plant combinations (Table 2). The effects of NaCl treatment on V_{cmax} and J_{max} were pronounced in A plants (with a significant difference compared to its control), whereas no effects were detected in A/A25. Interestingly $A_{N390,}$ g_s, V_{cmax} and J_{max} parameters were higher in A/A25 compared to A plants under control. Likewise, no effects on TPU were observed following salt stress in A/A25 and, once again, a significant reduction in the ungrafted A plants was observed (Table 2).

The maximum PSII quantum yield of primary photochemistry (F_v/F_m) did not change in both the genotypes following salinity stress, but showed values typical of healthy leaves (Björkman and Demmig, 1987) (*data not shown*). The ETRs for each plant combination subjected, or not, to salinity were plotted according to PAR (Fig. 3A, B). When PAR fell within the 0-200 μmol m^{-2} s^{-1} range in both plant types, the light-response curves of the ETR for the pepper-stressed plants closely overlapped that of the controls. Yet when PAR was above 200 μmol m^{-2} s^{-1}, in A-stressed plants, a significant separation of the light-response curves of ETR occurred (Fig. 3A). In A/A25 plants, the curves for control and salt did not show significant differences due to PAR (Fig. 3B).

The decrease in ETR in A salted plants was mainly caused by the substantial rise in NPQ (Fig. 3C). In A/A25 no differences in the NPQ values between the controls and treated plants were detected (Fig. 3D). The q_P coefficient remained

unchanged in A/A25 under salt stress (Fig. 3F), and lowered in A-stressed plants (Fig. 3E).

3.5. Antioxidant enzymes

SOD activity increased significantly in both genotypes following salinity (Fig. 4A), but the rise in A/A25 was even more pronounced. In A plants, CAT activity increased significantly following salinity conditions (Fig. 4B), whereas no changes in APX activity were recorded (Fig. 4C). A different behavior was observed in A/A25 plants, in which salt stress did not induce changes in CAT activity, but significantly reduced APX activity (Fig. 4B, C).

3.6. *Effect of salt treatment on lipid peroxidation, α-tocopherol, β-carotene, ascorbate and proline*

NaCl treatments led to a significant rise in the levels of the MDA by-products content in both kinds of pepper plants (Fig. 5A), but this increase was higher for A plants under salt stress. The α-tocopherol concentration (Fig. 5B) was also affected by NaCl treatment in A plants, whose a significant reduction was detected, but no differences were found between controls and treated plants for A/A25 (Fig. 5B). Another important antioxidant in chloroplast is β-carotene, which did not change in all plants following salt stress (Fig. 5C), even though a smaller amount was found in A/A25 compared to A plants (Fig. 5C). Finally, total AsA significantly increased in A plants under salinity conditions (+256% as compared to the controls). The decrease in the AsA/AsA total ratio in A salt-stressed leaves (from 0.85 to 0.52 in controls) indicated that a large amount of

AsA was oxidized into DHA (Fig. 5D). In A/A25 plants, a significant increase in the AsA/AsA total ratio was reported following the salinity treatment (Fig. 5D).

Proline content sharply increased, but only in A/A25 plants following NaCl stress, whereas no changes in A plants were observed (Fig. 6A).

3.2. Biomass

A/A25 plants developed a bigger root system than A plants (Fig. 7). No significant effect of salinity was noted on root FW and DW between the same plant types (Fig. 7A, C). The root FW/DW ratio did not change in both plant combinations (Fig. 7E).

A sharper drop in shoot biomass (leaf FW and DW) occurred as a consequence of salinity stress in A plants, but no changes in A/A25 were observed (Fig. 7B, D). On the contrary in A/A25, the FW/DW leaves ratio significantly lowered, but only in A/A25 (Fig. 7F).

4. Discussion

Under salinity stress, reduced plant growth is induced by different biochemical, physiological and molecular alterations (Munns, 2002; Krasensky and Jonak, 2012). The selection of salt tolerant accessions to be used as rootstocks could be a promising approach to ameliorate the negative effects of salinity on pepper productivity (Penella et al., 2013; Penella et al., 2015). In the present study, we demonstrated that Adige peppers grafted onto the accession A25 were less sensitive to salt stress compared to their ungrafted counterparts. The lower salt sensitivity exhibited by A/A25 was clearly demonstrated by the lack of negative effects on plant growth, increased marketable yield and the

fewer BER symptoms appearing. The ameliorative effect of grafting on plant's growth under salinity conditions fully agrees with other findings in tomato and melon (Santa-Cruz et al., 2002; Estañ et al., 2005; Martínez-Rodríguez et al., 2008; He et al., 2009).

According to Munns biphasic model (Munns and Tester, 2008), salt tolerance can be improved by reducing the negative osmotic effects on growth and/or maintaining leaf-root growth and functions for longer by diluting toxic ions (Balibrea et al., 2000; Yeo, 2007). Maintenance of shoot and root vigor is dependent mainly on photosynthetic capacity (Duarte et al., 2014; Penella et al., 2015). Photosynthetic activity remained unchanged in A/A25 plants under salt conditions compared to their controls and, therefore, also in the supply of photosynthates to plants, as confirmed by the absence of reduced plant growth. Conversely, the leaf CO_2 assimilation rate sharply dropped in the salt stressed A plants compared to both controls and A/A25 plants. Salt stress has been reported to reduce CO_2 assimilation through different mechanisms: (I) decreased stomatal conductance (Chaves et al., 2009; Shabala and Munns, 2012); (II) reduced mesophyll conductance to CO_2 (Flexas et al., 2004); and (III) impaired Rubisco activity (Galmes et al., 2013). Stomatal closure is certainly one of the main responses of plants under salinity to minimize water loss (Aroca et al., 2012; Shabala and Munns, 2012). Stomatal conductance decreased under salt treatment in both the A and A/A25 plants, which could be one of the reasons for their unchanged RWC values, and this suggests a typical conservative strategy (Tardieu and Simonneau, 1998; Garcia-Sánchez et al., 2010; Sade et al., 2012). Notably, in the grafted plants, the CO_2 assimilation rate did not change even if g_s significantly decreased under salinity conditions.

In contrast, in A plants the sharp reduction in g_s induced a marked decrease in A_{N390} (about -45%), to suggest that mesophyll limitations also occurred, as confirmed by the unchanged C_i. In fact, the unchanged intercellular CO_2 concentration was also likely attributable to the marked reduction in the V_{cmax} as observed in A plants. Other authors have reported that carboxylation efficiency under stress conditions is limited by the amount, activity and kinetics of Rubisco, as well as by an effect on CO_2 diffusion (Carmo-Silva and Salvucci, 2012; Koyro et al., 2013). The A/C_i curves also showed a significant decrease in J_{max} in A salt-treated plants and TPU, according to the Farquhar model (Farquhar et al., 1980), whereas no alterations were observed in grafted plants. These results suggest that carboxylation efficiency, ribulose-1,5-*bis*phosphate regeneration and triose-phosphate utilization were maintained in A/A25, whereas these processes were severely unpaired in A. There are some evidences in cucumber grafted plants (Liu et al., 2013) that the rootstock regulates the activity of Rubisco by the overexpression of *rbc*L *rbc*S and *rca* (Rubisco large and small subunit genes and Rubisco activase gene, respectively). The TPU rate has been proposed to at least provide an indication of the feedback between growth and CO_2 assimilation (Wullschleger, 1993). The sharp drop in A_{N390} in the A salt-treated plants related to the limitation in TPU can be considered one of the main reasons for reduced growth (Long and Bernacchi, 2003; von Caemmerer, 2000; Sharkey et al., 2007).

Leaves of grafted plants displayed higher values of water use efficiency (WUE) compared with leaves of ungrafted plants (3.98 *versus* 3.39 µmol CO_2/mmol H_2O) following salt treatment and showed a better stomatal control and higher carboxylation efficiency (in A/A25 45% more than in A plants).

Clearly, the lower constitutive value of V_{cmax} coupled with the step decrease following salt treatment was probably responsible to a decrease CO_2 concentration at chloroplastic level in A plants. The increase in WUE in grafted plants following salinity results from an improvement of photosynthetic rate. On the other hand, A25/A plants have a more developed biomass root system than A plants and this was not modified under salt treatment. Higher root development in grafted plants could be the consequence of the higher photosynthetic rate determined in grafted plants independently to salt stress. This indicates that carbohydrates produced by photosynthesis are translocated to the root where they are utilized for respiration and growth. Consequentially, a balance between root absorption and photosynthesis would result.

The stomatal and biochemical limitations imposed on photosynthesis in A plants submitted to the salt treatment were likely accompanied by a lowered ATP and NADPH consumption rate for CO_2 assimilation, which would imply a lower ETR (Baker and Rosenqvist, 2004). A progressive drop in ETR can be compensated by an increased thermal dissipation (Medrano et al., 2002). Accordingly, NPQ increased once A plants were subjected to salinity, even though they underwent higher excitation pressure on PSII and more reaction centers were closed, as evidenced by an over-reduction of Q_A (Calatayud and Barreno, 2001; Guidi and Calatayud, 2014; Kalaji et al., 2014). This is particularly evident at high light (800-1000 µmol photons m^{-2} s^{-1}) when salt stress can accelerate photodamage to the reaction center of PSII (Nishiyama and Murata, 2014). Even though the actual PSII efficiency was compromised, the dissipation mechanisms were able to preserve PSII to irreversible damage, and the F_v/F_m values remained unchanged. Conversely, the chlorophyll

fluorescence parameters in the A/A25 salt-treated plants confirmed that no alterations occurred in the biochemical and photochemical chloroplast processes, as previously revealed by gas exchange analyses. These results coincide with previous findings, which highlighted that the use of tolerant rootstock improved the photosynthesis performance of the scion under salinity conditions (Moya et al., 2002; Massai et al., 2004; He et al., 2009; Penella et al., 2015).

Although the marked accumulation of toxic ions occurred in the A/A25 plants similarly to A plants subjected to salinity, no effects were detected in photosynthesis of A/A25 and the antioxidant systems were not activated in grafted plants, except for SOD activity. In addition to this enzyme, the activity of the primary antioxidant enzymes involved in removing and/or scavenging ROS (CAT and APX) were significantly stimulated in the A plants under salinity. The incremented activities of these enzymes and/or the stimulated biosynthesis of antioxidant molecules have long been described as being actively involved in response to several abiotic stresses, including salt toxicity in both grafted and ungrafted plants (López-Gómez et al., 2007; He et al., 2009; Sanchez-Rodríguez et al., 2012; Shaheen et al., 2013). In this context, it is assumed that the simultaneous involvement of antioxidant components is necessary to obtain an increase (and/or a faster response) in plant defenses when plants face high salinity (Jaleel et al., 2009). However in the A plants, the antioxidant system did not efficiently sustain ROS scavenging in relation to salinity-triggered ROS production, as demonstrated by the marked increase in the MDA by-product levels.

Total AsA increased significantly (about 44% compared to the controls) in the A plants under salinity, and the AsA/(DHA+AsA) ratio also sharply dropped, which indicates that a high AsA oxidation rate occurred. No differences were observed in the total AsA and AsA/(DHA+AsA) ratio in the A/A25 plants under salt stress compared to their controls. Despite the increase in the amount of DHA found in A plants under salinity, oxidation of AsA was not sufficient to efficiently sustain the α-tocopherol regeneration rate being ascorbate essential for α-tocopherol regeneration (Szarka et al., 2012). The fail of this biochemical mechanism can further increased membrane lipid peroxidation, as revealed by the dramatic increase in the MDA by-products level in A plants under stress.

Accumulation of osmolytes, such as proline, is a well-known adaptive mechanism in plants against salt stress conditions (Ashraf and Foolad, 2007; Szabados and Savouré, 2010). Several studies have attributed a dual role to proline: compatible osmolyte and antioxidant compound (Szabados and Savouré, 2010). It has been previously reported that under salt stress proline can contribute by stabilizing many functional units, such as Complex II in the mitochondrial electron transport chain and key enzymes, such as Rubisco (Ashraf et al., 2008). In A/A25 leaves, proline content increased 2.6-fold in the presence of NaCl excess, compared to a non-significant increase noted in A leaves.

Overall, our results suggest that A/A25 plants were tolerant to the salt concentration adopted in this experiment given the adjustments made in the physiological processes and starting from a more abundant root biomass production (compared to ungrafted plants). Despite it is undeniable that the roots play an important role in determining the salt tolerance of the scion, little is

known about how the scion was influenced by the rootstock (A25) in this experiment. Grafting has been described to increase salt tolerance by excluding or restricting ion toxic accumulation in the shoot (Colla et al., 2013). In fact, we previously reported this mechanism also in pepper by using different salt-tolerant rootstocks (Penella et al. 2015). Differently, in the present experiment A/A25 plants accumulated high concentration of toxic ions in their tissues, as also reported by He et al (2009) in salt-tolerant grafted tomato plants. The increase of Na^+ and Cl^- into plant tissues and their probable compartmentalization in the vacuole, determine the accumulation of compatible solutes in the cytosol and organelles to balance the osmotic pressure (Munns and Tester, 2008). The increased proline levels suggest that this osmolyte could act in that way, thus preserving enzymatic activities or protein stability in accordance to previous findings (Gupta and Huang, 2014; Zrig et al., 2016).

The higher SOD activity observed in A/A25 plants under salinity, leading to H_2O_2 production, could be the signal triggering the cascade of adaptive (genetic and physiological) responses (Bose et al., 2014, Rejeb et al., 2015). Recently, several researches have indicated that proline accumulation occurs in stressed plants and can be mediated by signaling molecules, including H_2O_2 (e.g. Zhu, 2002; Zhang et al., 2008; Yang et al., 2009; Wen et al., 2013). In this context, it may be speculated that proline is the key metabolite by which A/A25 plants tolerated the salinity conditions imposed in the present experiments. However, with our results it is not allowed to speculate whether the SOD-triggered proline biosynthesis is a specific prerogative of A25. In addition, beyond the key role of proline, other mechanisms not contemplated in this work can have further

improved the salt tolerance of grafted plants, thus making the topic warrant of future investigation.

To conclude, grafting of commercial variety onto salt-tolerant rootstocks can be considered a valid strategy for ameliorating the salt tolerance of pepper as testified by the larger amount of marketable fruits and the lower BER incidence in the A/A25 plants grown under field whose soil was affected by moderate salinity.

Acknowledgments

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Table 1. Marketable fruit yield and percentage of fruit affected by Blossom end Root (BER) under water and soil salinity conditions. Values are the mean of 50 replicates *per* cultivar Adige ungrafted (A) or grafted onto the A25 genotype (A/A25). Different letters in each column indicate significant differences at $P<0.05$ using the LSD test, following a one-way ANOVA test with plant type as the variability factor

Graft combination	Marketable yield (kg plant^{-1})	BER (%)
A	1.84 b	49a
A/A25	3.23 a	18b

Table 2. Gas exchange parameters of cultivar Adige ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions. Plants maintained in optimal nutrient solution represent the controls. The CO_2 assimilation rate at 390 µmol mol^{-1} CO_2 (µmol CO_2 mol^{-1}) (A_{N390}), the intercellular CO_2 concentration (µmol CO_2 mol^{-1}) (C_i) and stomatal conductance to water vapor (mol H_2O m^{-2} s^{-1}) (g_s) were determined from the response curve of the CO_2 photoassimilation *versus* light intensities. The apparent maximum carboxylation rate of Rubisco (V_{cmax}, µmol CO_2 $m^{-2}s^{-1}$), the maximum rate of electron transport (J_{max}, µmol e^- m^{-2} s^{-1}), which is the equivalent to the RuBP regeneration rate, and the use of triose-P (TPU; µ Pi $m^{-2}s^{-1}$) were determined from response curve of CO_2 photoassimilation *vs.* C_i. Values are the mean of four replicates *per* plant combinations and treatment. Different letters in each column indicate significant differences at $P<0.05$ using the LSD test, following a two-way ANOVA test with NaCl treatment and plant type as the variability factors.

Graft combination	Treatment	A_{N390}	Ci	gs	Vcmax	Jmax	TPU
A	control	6.91 b	221.0 a	0.092 b	64.5 b	71.5 b	4.75 a
	NaCl	3.76 c	210.5 ab	0.035 c	31.0 c	44.0 c	2.40 b
A/A25	control	9.45 a	214.0 ab	0.135 a	124.0 a	103.5 a	5.55 a
	NaCl	8.18 ab	179.0 b	0.082 b	137.0 a	99.5 a	4.60 a

Figure captions

Fig. 1. Mineral content (on a DW basis) in the roots and leaves of the control (white bars) and salt-treated plants (black bars) of pepper cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25). Means (*n*=6; ± SE) with different letters being significantly different at *P*≤ 0.05 according to a two-way ANOVA, with salt treatment and plant type as the variability factors.

Fig. 2. Water potential and RWC (inside) of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Means (*n*=6 ± SE) with different letters are significantly different at *P*≤ 0.05 according to a two-way ANOVA, with salt treatment and plant type as the variability factors. Absence of letters (inside box) indicates that the F ratio was not significant.

Fig. 3. Electron transport rate (ETR), non-photochemical quenching (NPQ) and photochemical quenching coefficient (q_P) in response to photosynthetic active radiation (PAR) in cultivar Adige, ungrafted (A) or grafted onto the A25 accession (A/A25) under salinity conditions (closed circles). The plants maintained in optimal nutrient solution represent controls (open circles). Values are the mean of 6± SE replicates *per* plant combination.

Fig. 4. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activity in leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Means (*n*=6; ± SE) with different letters are significantly different

at *P*≤ 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

Fig. 5. Malondialdehyde by-products (MDA), α-tocopherol, β-carotene and ascorbic acid in the leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 accession (A/A25) under salinity conditions (black bar). Control is represented by white bars. In graph D, different forms of ascorbate are reported. The numbers above the bars indicate the AsA/AsA total ratio and capital letters indicate the difference. Means (*n*=6 ±SE) with different letters are significantly different at *P*≤ 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

Fig. 6. Proline content in the leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Each value represents the mean of 6 samples ± SE. Means with different letters are significantly different at *P*≤ 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

Fig. 7. FW and DW, and their ratio for the root and leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Each value represents the mean of 6 samples ± SE. Means with different letters are significantly different at *P*≤ 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors. Absence of letters indicates that the F ratio of the interaction is not significant.

Figure 1

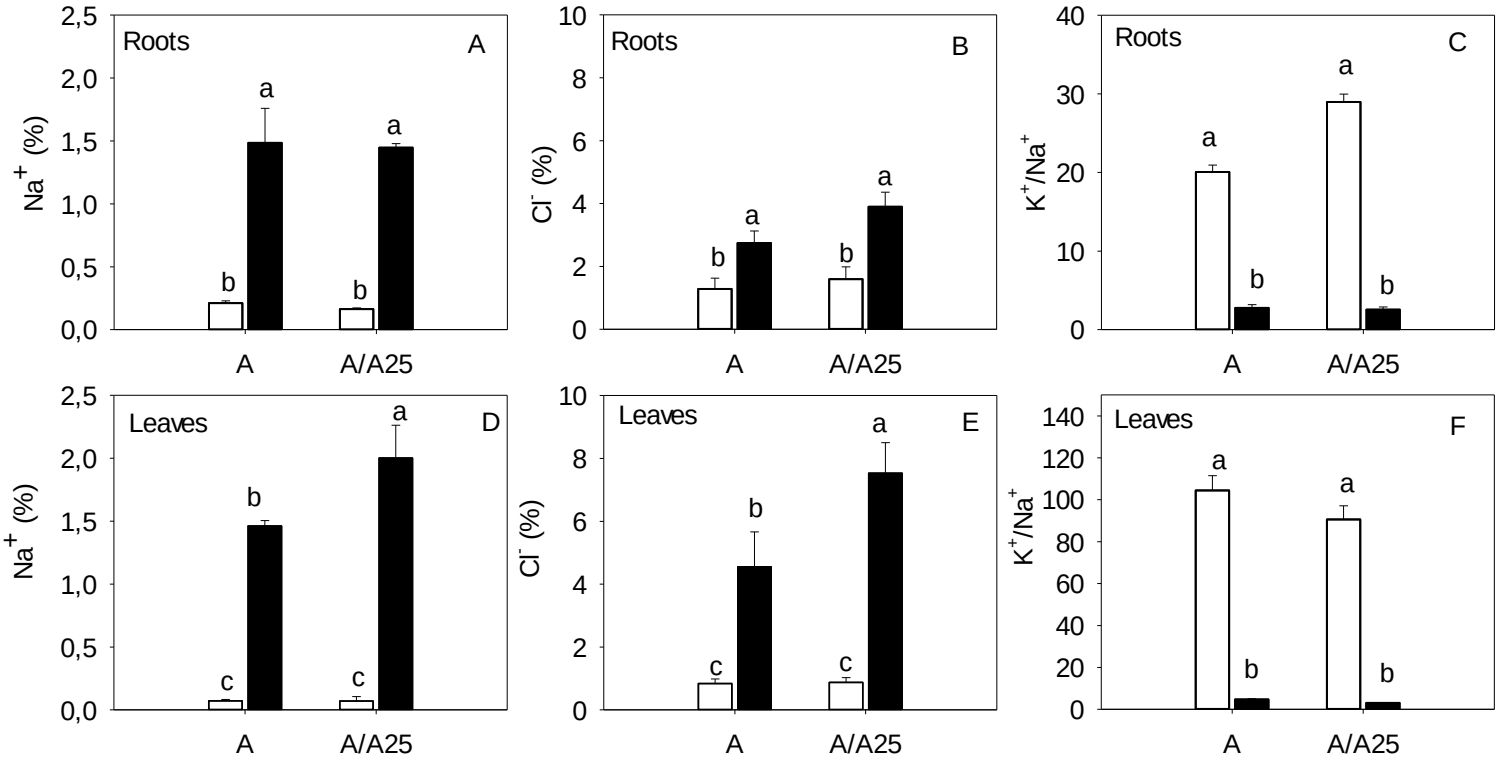

Roots
A
Na+ (%)
2,5
2,0
1,5
1,0
0,5
0,0
a
a
b
b
A
A/A25
Roots
B
Cl- (%)
10
8
6
4
2
0
a
a
b
b
A
A/A25
Roots
C
K+/Na+
40
30
20
10
0
a
a
b
b
A
A/A25
Leaves
D
Na+ (%)
2,5
2,0
1,5
1,0
0,5
0,0
a
b
c
c
A
A/A25
Leaves
E
Cl- (%)
10
8
6
4
2
0
a
b
c
c
A
A/A25
Leaves
F
K+/Na+
140
120
100
80
60
40
20
0
a
a
b
b
A
A/A25

Figure 2

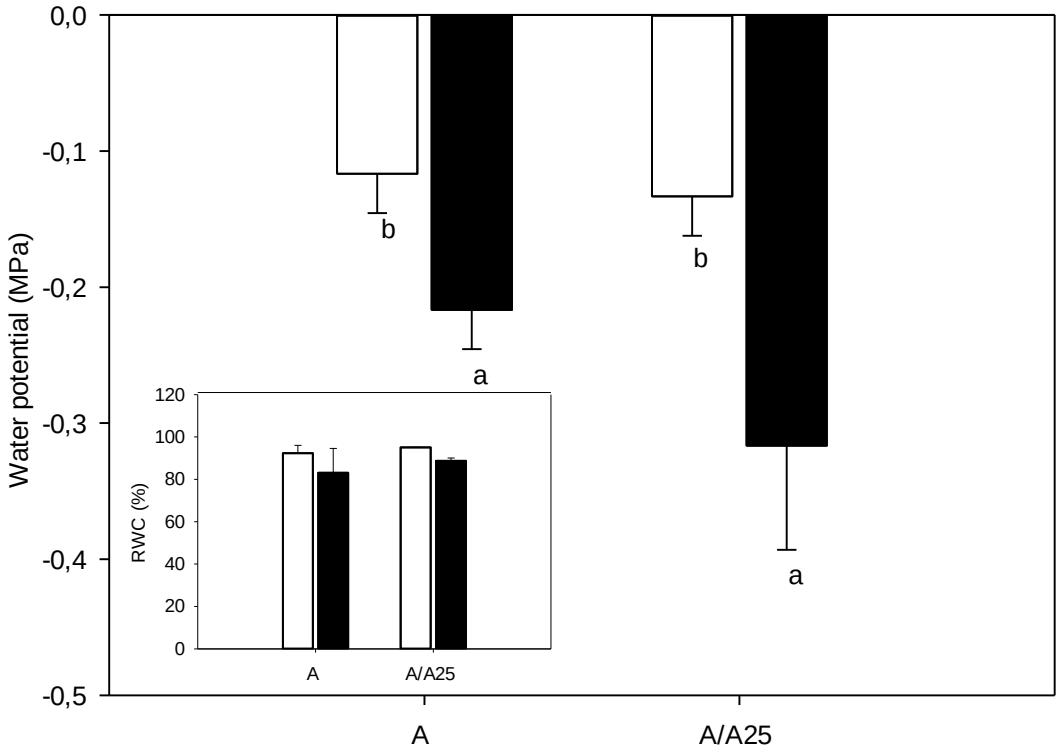

0,0
-0,1
-0,2
-0,3
-0,4
-0,5
Water potential (MPa)
b
a
b
a
A
A/A25
RWC (%)
120
100
80
60
40
20
0
A
A/A25

Figure 3

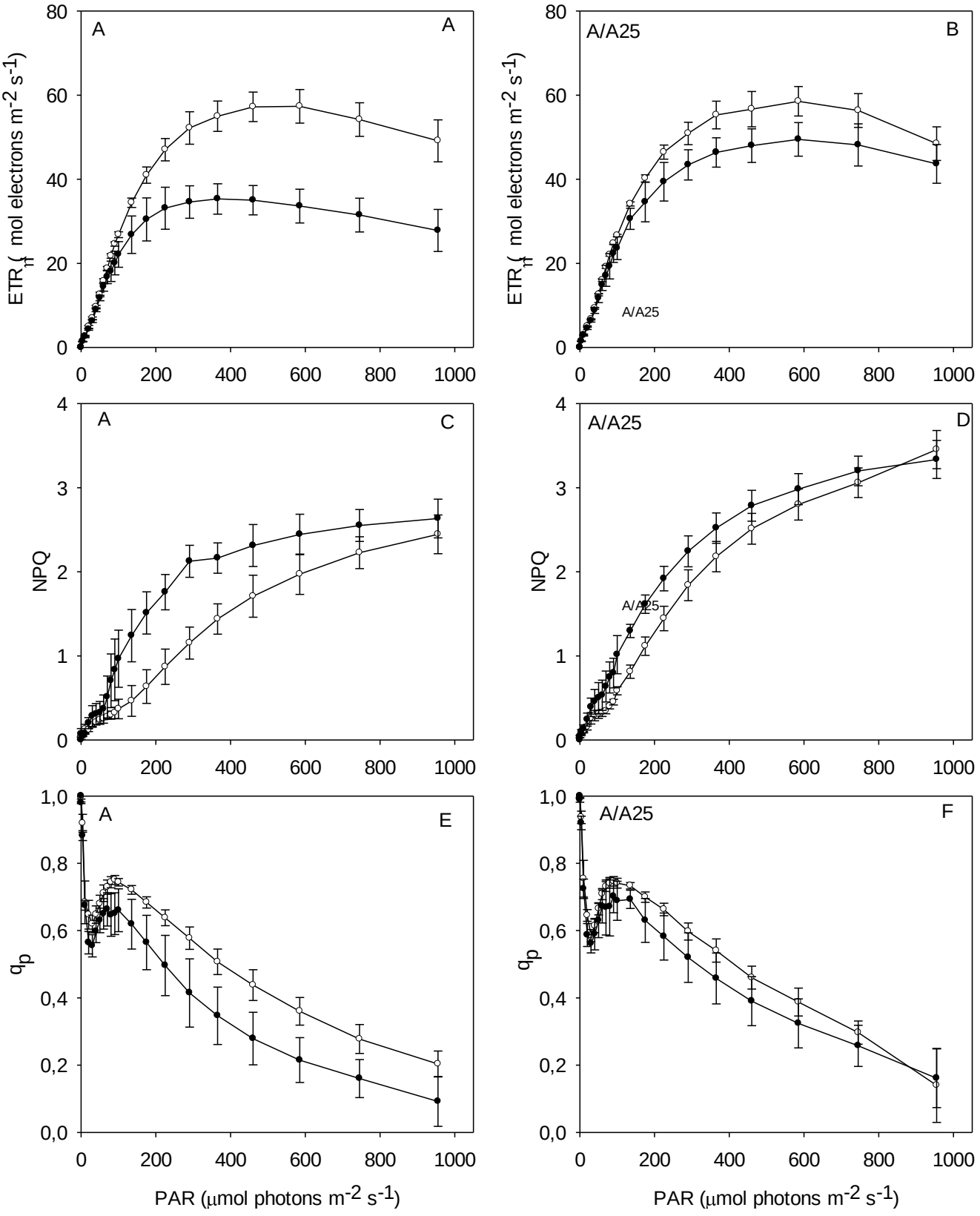

A
A
A/A25
B
A
C
A/A25
D
A
E
A/A25
F
ETR_II (mol electrons m-2 s-1)
NPQ
qp
PAR (µmol photons m-2 s-1)
PAR (µmol photons m-2 s-1)

Figure 4

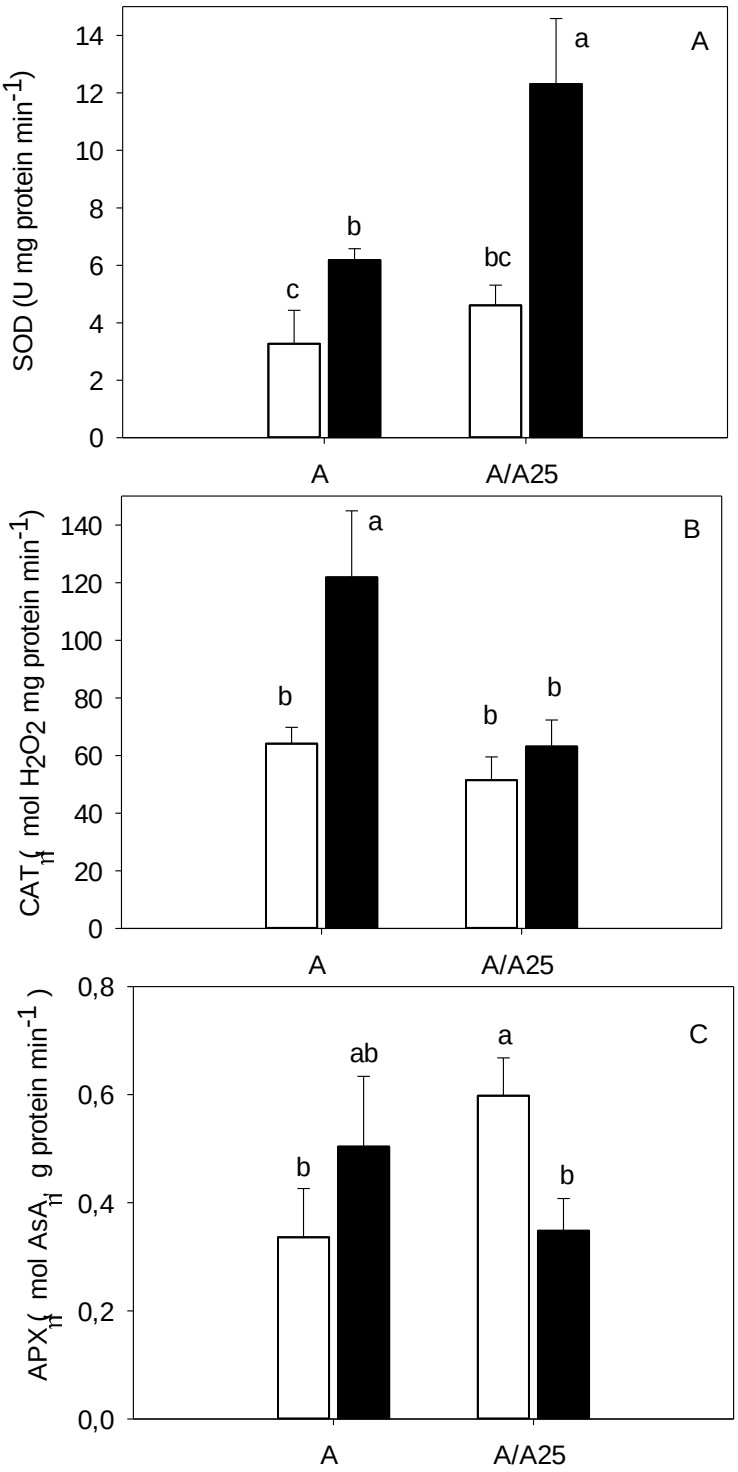

A
SOD (U mg protein min-1)
14
12
10
8
6
4
2
0
a
b
c
bc
A
A/A25
B
CAT (mol H2O2 mg protein min-1)
140
120
100
80
60
40
20
0
a
b
b
b
A
A/A25
C
APX (mol AsA g protein min-1)
0,8
0,6
0,4
0,2
0,0
ab
a
b
b
A
A/A25

Figure 5

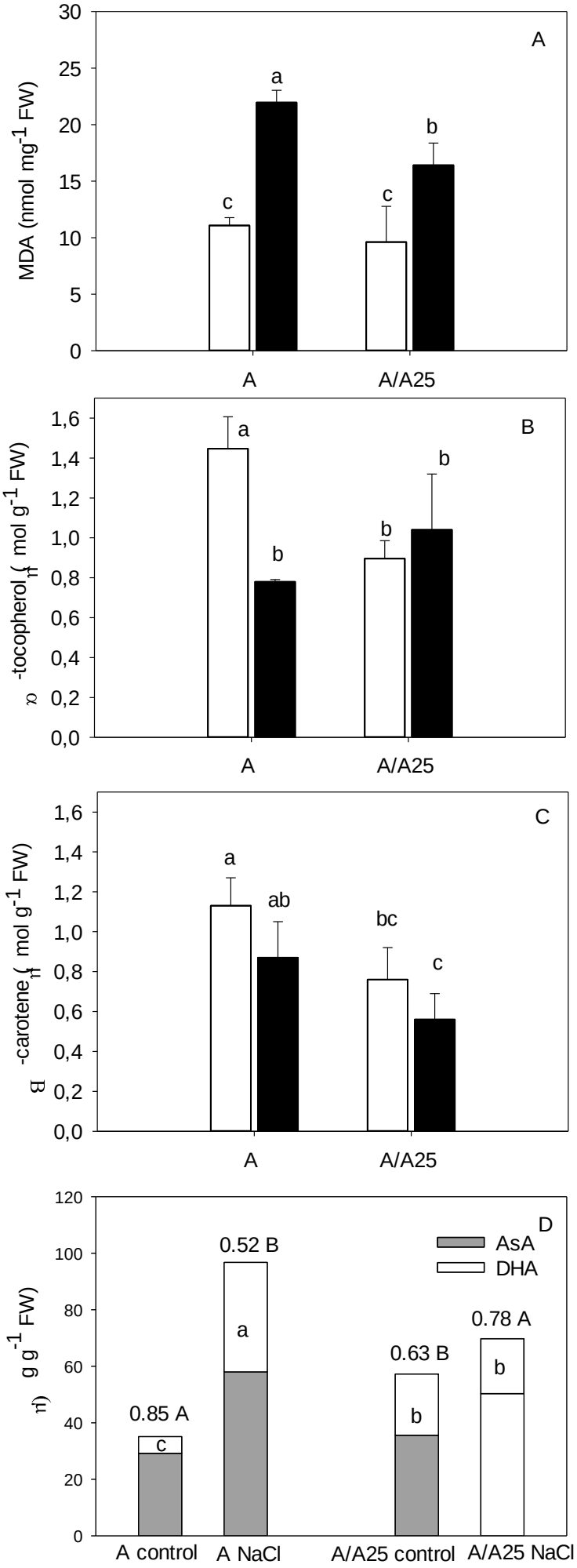

A
MDA (nmol mg-1 FW)
30
25
20
15
10
5
0
a
b
c
c
A
A/A25
B
-tocopherol (mol g-1 FW)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
b
b
b
A
A/A25
C
-carotene (mol g-1 FW)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
ab
bc
c
A
A/A25
D
AsA
DHA
g g-1 FW)
120
100
80
60
40
20
0
0.52 B
a
0.85 A
c
0.63 B
b
0.78 A
b
A control
A NaCl
A/A25 control
A/A25 NaCl

Figure 6

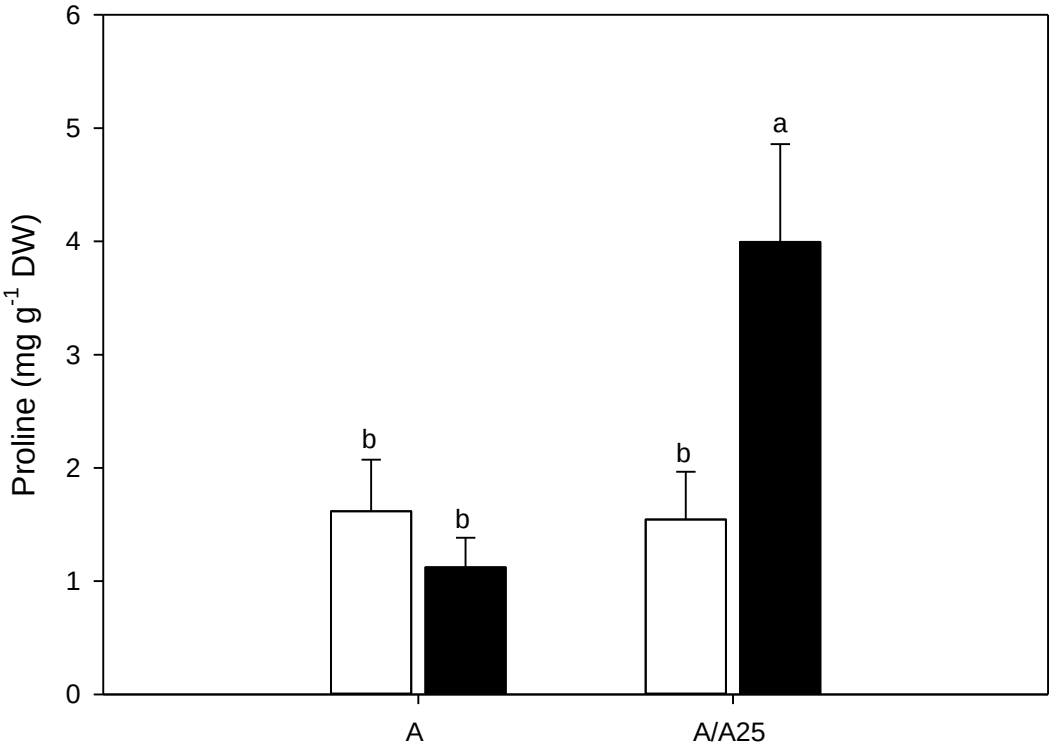
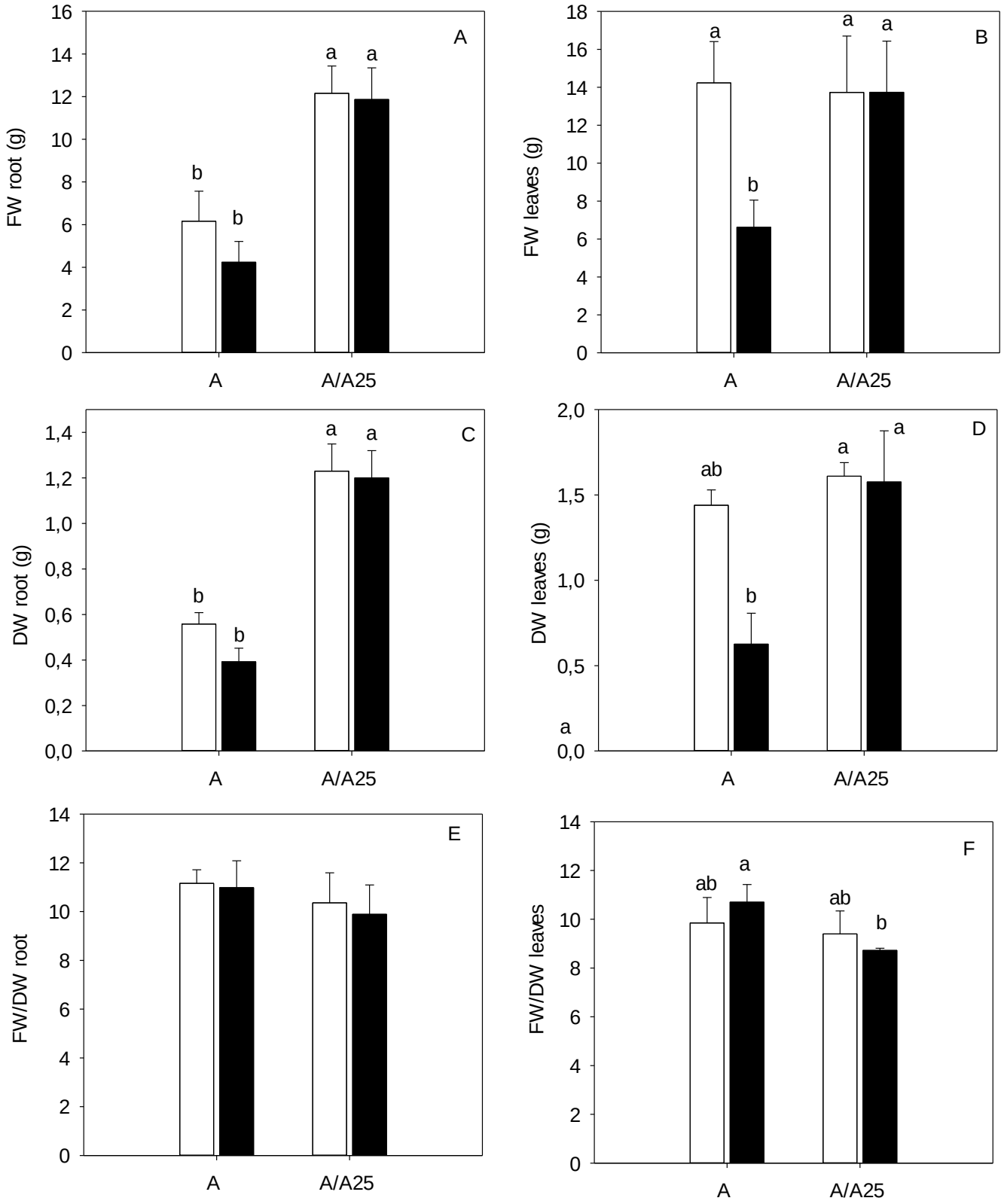


Figure 7


A
FW root (g)
16
14
12
10
8
6
4
2
0
b
b
a
a
A
A/A25
B
FW leaves (g)
18
16
14
12
10
8
6
4
2
0
a
b
a
a
A
A/A25
C
DW root (g)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
b
b
a
a
A
A/A25
D
DW leaves (g)
2,0
1,5
1,0
0,5
0,0
a
ab
b
a
a
A
A/A25
E
FW/DW root
14
12
10
8
6
4
2
0
A
A/A25
F
FW/DW leaves
14
12
10
8
6
4
2
0
ab
a
ab
b
A
A/A25